REMARKS

Claims 1, 4-11, 14-16, 18-26, and 29-39 are currently pending in the application.

Claims 1 and 16 have been amended herein to clarify the nucleotide sequence and its complementarity. The specification and Sequence Listing have also been amended for this purpose. The Sequence Listing has also been amended to add SEQ ID NO: 7, which was previously present in the specification but was not listed in the Sequence Listing. Support for these amendments can be found throughout the specification. Accordingly, no new matter has been added by these amendments.

The specification has also been amended to insert appropriate SEQ ID NO identifiers that were previously not included in the specification. Accordingly, no new matter has been added by this amendment.

Applicant again notes that the Attorney Docket Number for this application has changed to **HYZ-069CN/ 47508.530**.

In addition, Applicant notes that contrary to the assertion at page 7 of the Office Action, this application does not name joint inventors.

The outstanding rejections are addressed individually below.

1. ***Claims 14, 15, and 34-36 are enabled.***

Claims 14, 15, and 34-36 stand rejected under 35 U.S.C. § 112, first paragraph, for allegedly lacking enablement over the scope claimed "because the specification, while being enabling for inhibiting HIV-1 or HIV-2 infection in a cell *in vitro* does not reasonably provide enablement for inhibiting HIV infection in a cell *in vivo*. Applicant respectfully traverses this rejection.

First, the claimed embodiments of the invention are enabled because they are neither overly broad in scope, nor require undue experimentation. In applying the *Wands* factors to an assessment of enablement of the instant claimed embodiments of the invention, the Office Action states that the "quantity of experimentation required to

practice the invention as claimed would require the *de novo* determination of accessible target sites, modes of delivery and formulations to target appropriate cells and/or tissues harboring HIV-1 or HIV-2 whereby HIV replication and infection is inhibited in cells *in vivo*." (Office Action, page 6)

Applicant respectfully notes that, not only is the breadth of the claims in the instant application not very broad at all, but also that the amount of experimentation required is not undue. Indeed, the claimed embodiments of the invention in dispute are limited to oligonucleotides that inhibit HIV-1 or HIV-2 infection in a cell or which exhibit antiviral activity against HIV-1 or HIV-2, or to methods of inhibiting HIV-1 or HIV-2 infection in a cell using an antisense oligonucleotides having only a few different nucleotide sequences (*e.g.*, the possible 21mer sequences within the 22mer sequence specified by SEQ ID NO: 5 including, without limitation, SEQ ID NOs: 1 and 3). Furthermore, such sequences have been shown to be effective in inhibiting and preventing HIV-1 infection in MT-4 cells (see, *e.g.*, Figures 1-3 which show the efficacy of "GEM® 92" (*i.e.*, SEQ ID NO: 1) for inhibition of HIV-1 infection or to inhibit HIV-2-induced cell killing) Additionally, some clinical trials have also been performed (see Exhibits B and C). Accordingly, there would also be no undue experimentation required in using the claimed methods. Therefore both the breadth of the claims and the quantity of experimentation required supports enablement of the claimed embodiments of the method of the invention.

Second, the Office Action states, in further applying the *Wands* factors to an assessment of enablement of the claimed invention, that "the in vivo (whole organism) application of nucleic acids (such as antisense) is a highly unpredictable endeavor due to target accessibility and delivery issues," citing Branch ((1998) TIBS 23: 45-50) and Crooke (1998) Antisense Research and Application pp. 1-50). The Office Action further states that "[t]he high level of unpredictability regarding the prediction of antisense efficacy in treating disease states was illustrated in the clinical trial results obtained by ISIS pharmaceuticals for the treatment of Crohn's disease using antisense targeting

ICAM-1, whereby the placebo treatment was found more successful than antisense treatment." (Office Action, pages 3-4)

Applicant notes that the claimed embodiments of the invention in this case have overcome the cited unpredictability in the art. Indeed the cited problem of "target accessibility" has been overcome, as evidenced by the support in the specification showing efficacious anti-HIV effects of the claimed antisense in infected MT-4 cells, while the cited issue of "delivery" is not problematic as evidenced by the *in vivo* studies cited above and discussed further below. Furthermore, while there may be individual and specific instances of failure that support the assertion that the use of antisense *in vivo* "is a highly unpredictable endeavor due to target accessibility and delivery issues," they are not determinative of enablement of the instant claimed embodiments of the invention. In particular, Applicant respectfully notes that an isolated statistical failure in a phase III clinical trial required for FDA approval does not defeat patentability for lack of enablement because the standards applied for FDA approval are not those required for patentability. The Federal Circuit has stated that "considerations made by the FDA for approving clinical trials are different from those made by the PTO in determining whether a claim is enabled." (*See* MPEP 2164.05) Moreover, the cited negative report, published in 1999, is contradicted by subsequent positive phase II clinical trial results, which demonstrated "significant and long-lasting improvement of symptoms in patients with ulcerative colitis" (see Exhibit A).

Still further, Applicant respectfully notes that the reasoning of the rejection is inconsistent, inasmuch as the cited results are for a different antisense oligonucleotide that targets a different mRNA for use in treating a different disease. Even if the cited anti-ICAM-1 antisense did fail, it would not negate patentability of the instant anti-HIV antisense oligonucleotides. Furthermore, the failure of one individual antisense oligonucleotide does not logically support lack of enablement of completely unrelated antisense oligonucleotides with different targets and disease indications. Indeed, while Applicant can cite numerous instances of success with unrelated antisense

oligonucleotides that have been shown to be effective *in vivo*, it is the success of the claimed anti-HIV antisense oligonucleotides that is most relevant to the question of enablement in this case.

The success of anti-HIV antisense oligos related to the instant claimed embodiments of the invention is evident from the results of numerous *in vivo* studies which follow the teachings of the specification, which are discussed further below. For example, an initial single dose phase 1 study of GEM®92, corresponding to SEQ ID NO:1 of the instant application, administered orally at three dose levels as well as by injection showed excellent safety results and demonstrated effective oral delivery in humans of these 2nd generation antisense agents (see Exhibit B, page 2 from Applicant's website at www.hybridon.com/drugdevelop/ clinical_program). Furthermore, Applicant's previous anti-HIV antisense treatment, GEM®91, which corresponds to a 25-mer phosphorothioate oligonucleotide that targets the same *gag* sequence as the instantly claimed antisense oligonucleotides "significantly reduced viremia in HIV-positive patients treated for up to eight days" (see Exhibit C). Although concerns regarding the safety of GEM®91 may hinder FDA approval or further commercial development of this 25mer, as discussed above, they do not negate patentability of the instant related antisense technology.

Finally, the Office Action states, in still further applying the *Wands* factors to an assessment of enablement of the instant claimed invention, that "Applicants have not provided guidance in the specification toward a method of inhibiting HIV-1 or HIV-2 infection in an organism." (Office Action, page 5) Further, the Office Action states that the specification "fails to provide any particular guidance for the targeting and inhibition of appropriate target cells harboring HIV or susceptible to HIV-1 or HIV-2 infection . . . and since determination of these factors is highly unpredictable, it would require undue experimentation to practice the invention over the scope claimed." (Office Action, page 6) Applicant respectfully disagrees and points to the extensive

teachings of the specification that support enablement, which requires that the application teach how to make and use the invention.

First, the specification does teach one of skill in the art to how to make the invention (*see, e.g.*, the specification at page 13, line 26 to page 15, line 30 and Example 1, page 32, line 13 to page 33, line 2). Furthermore the specification teaches one of skill in the art how to use the invention (*see, e.g.*, the specification at page 20, line 10 to page 21, line 30 (describing pharmaceutical formulations), page 21, line 32 to page 24, line 8 (describing therapeutically acceptable methods and amounts), page 24, lines 10-20 (describing methods of administration), and page 24, line 22 to page 26, line 24 (describing therapeutic formulations and pharmaceutical compositions)). Accordingly, the specification has fully enabled the invention as claimed because it teaches how to make and use the invention without undue experimentation.

Applicant further notes that the specification provides numerous examples supporting enablement. For example, the oligonucleotides of the invention have been tested extensively *in vitro* in a variety of cell types. These *in vitro* experiments were performed by analyzing the ability of the claimed antisense oligonucleotides to inhibit existing infections and to protect against infection in MT-4 cells (page 19, lines 18-25). The results of these experiments are shown in Figures 1 and 2. Additional *in vitro* experiments include an analysis of the preclinical range of anti-HIV activity of various oligonucleotides of the invention (page 26, line 26 to page 30, line 8), and an analysis of toxicity in fresh human peripheral blood mononuclear cells (page 30, lines 10-28). Several of these experiments are also detailed in the Examples 7 and 8 (page 42, line 13 to page 47, line 27), which describe testing the anti-HIV activity of the claimed antisense oligonucleotides in fresh human peripheral blood lymphocytes and fresh human monocyte-macrophages as well as inhibition of acute infection of MT-4 cells

Additionally, Applicant respectfully emphasizes that these *in vitro* results support enablement of the claimed *in vivo* methods. M.P.E.P § 2164.02 states:

> [a]n *in vitro* or *in vivo* animal model example in the specification, in effect, constitutes a "working example" if that example "correlates" with a disclosed or claimed method invention. . . . In this regard, the issue of "correlation" is also dependent on the state of the prior art. In other words, if the art is such that a particular model is recognized as correlating to a specific condition, then it should be accepted as correlating unless the examiner has evidence that the model does not correlate.

This section further states that a "rigorous or an invariable exact correlation is not required . . . ." In the instant case, the specification provides extensive support for, and evidence of, the efficacy of the invention *in vivo*. For example, at page 31, line 23 to page 32, line 5, and Example 9 (page 47, line 29 to page 52, line 23) of the instant patent application provides a description of *in vivo* testing methods to evaluate the presence of the claimed antisense oligonucleotides absorbed by the body after oral administration. In addition, the specification, at page 30, line 30 to page 31, line 21, teaches that oligonucleotides of the invention, when examined *in vivo*, were found to be intravenously and orally bioavailable in both rats and monkeys. Indeed, the specification, as amended, states at page 30, line 30 to page 31, line 4 that "the bioavailability of Oligo 12 was examined *in vivo* and was found to be intravenously and orally bioavailable to rats and monkeys after a single dose. . . . [and] synthetic oligonucleotides systemically administered to pregnant murine females were found to cross the placenta and be available in the blood of embryos *in utero*." Furthermore, the specification at page 31, lines 14-21 states that an oligonucleotide of some embodiments of the invention "was found to be absorbed through the gatstrointestinal tract and accumulated in various organs and tissues" following intravenous or oral administration.

Finally, in support of enablement of the claimed invention, Applicant has submitted Exhibits B and C, discussed above, which discuss some of the results from various human clinical trials.

In conclusion, Applicant asserts that the claimed invention is enabled because of the relative narrowness of the claims, the state of development of the claimed invention, and the extensive guidance provided in the specification showing how to make and use the invention without undue experimentation. Accordingly, reconsideration and withdrawal of the enablement rejection is respectfully requested.

2. ***Claims 1, 4, 5, 8, and 9 are not anticipated by Agrawal et al.***

Claims 1, 4, 5, 8 and 9 stand rejected under 35 U.S.C. § 102(a) or (e) as being anticipated by Agrawal *et al.* Applicant respectfully traverses this rejection.

M.P.E.P. § 2131 quotes that a "claim is anticipated only if each and every element as set forth in the claim is found, either expressly or inherently described, in a single prior art reference."

Independent claim 1 recites a synthetic oligonucleotide comprising a nucleotide sequence specifically complementary to nucleotides 324 to 345 of a conserved *gag* region of the HIV-1 genome set forth as SEQ ID NO:5, the oligonucleotide consisting of 21 nucleotides. The nucleotides are linked via phosphorothioate internucleotide linkages, the oligonucleotide comprises at least two 5'-terminal ribonucleotides, or at least two 3'-terminal and at least two 5' terminal ribonucleotides, and the ribonucleotides are 2'-substituted ribonucleotides.

Applicant does not necessarily agree with the characterization of Agrawal *et al.* as described in the instant Office Action. However, Applicant does agree that, as stated by the Examiner in a previous Office Action, dated August 28, 2002, "Agrawal et al. do not teach the antisense oligonucleotides consisting of SEQ ID Nos: 1-4, which are 21 nucleobases in length and specifically target nucleotides 324-345 of gag of the HIV-1 genome as set forth as SEQ ID NO: 5." Applicant notes that the oligonucleotides in Table 1 of Agrawal *et al.* are each 25 nucleotides in length, not 21 nucleotides in length as required by Applicant's claimed embodiments of the invention.

Thus, the cited reference, Agrawal *et al.*, does not teach each and every element of claim 1.

Likewise, claims 4, 5, 8, and 9 are dependent (either directly or indirectly) on claim 1 and thus include all of the elements of that claim. Accordingly, these claims also do not teach each and every element of Agrawal *et al.*

Accordingly, Applicant respectfully submits that a *prima facie* case of anticipation has not been made. Applicant respectfully requests that the Examiner reconsider and withdraw this rejection.

3. ***Claims 1, 4, 5-11, 14-16, 18-26 and 29-39 are not obvious over Agrawal et al. in view of Hovanessian et al. and Goodchild et al.***

Claims 1, 4, 5-11, 14-16, 18-26, and 29-39 stand rejected under 235 U.S.C. § 103(a) as allegedly being unpatentable over Agrawal *et al.* in view of Hovanessian *et al.* and Goodchild *et al.* Applicant respectfully traverses this rejection.

At the outset, Applicant respectfully submits that a rejection over these references had been previously addressed by Applicant and was withdrawn by the Examiner as indicated in the Office Action dated December 19, 2003, which indicated that any rejections not repeated in that Office Action had been withdrawn.

Applicant respectfully submits that neither Hovanessian *et al.* nor Goodchild *et al.* cures the deficiency of Agrawal *et al.* recited above with regard to the rejected claims.

The specification of the instant application indicates at page 11, line 24 to page 12, line 11, that

> Novel antisense oligonucleotides have been designed which inhibit HIV-1 and HIV-2 replication. These oligonucleotides are synthetic oligonucleotides having phosphorothioate internucleotide linkages and a nucleotide sequence that is complementary to a portion of the *gag*

> region of the genome of HIV-1 and HIV-2. Sequences situated in this region have been demonstrated to be essential for viral packaging. . . . The oligonucleotides of the invention have been designed to bind to this region of RNA and DNA, thereby disrupting its natural stability and resulting ultimately in the inhibition of viral packaging and translation of *gag* mRNA. The specific sequence to which the oligonucleotides of the invention are complementary is nucleotides 324-345 of the *gag* region of HIV-1. This sequence is very conserved among strains of HIV-1, as shown below in TABLE 1.

This choice of these specific 21 nucleotide sequences was made for a specific purpose not suggested by Agrawal *et al.* Furthermore, Agrawal *et al.* does not even indicate that the SEQ ID NO: 1 disclosed therein is complementary to an HIV sequence such as the *gag* sequence. Therefore, one of skill in the art would not have been motivated to specifically use the oligonucleotides claimed in the instant application. Neither Hovanessian *et al.* nor Goodchild *et al.* cures the deficiency of Agrawal *et al.* recited above with regard to the rejected claims. Therefore even if combined, Agrawal *et al.* in view of Hovanessian *et al.* and Goodchild *et al.* does not teach all of the limitations of the claims.

As discussed above, independent claim 1 recites a synthetic oligonucleotide comprising a nucleotide sequence specifically complementary to nucleotides 324 to 345 of a conserved *gag* region of the HIV-1 genome set forth as SEQ ID NO:5, the oligonucleotide consisting of 21 nucleotides. The nucleotides are linked via phosphorothioate internucleotide linkages, the oligonucleotide comprises at least two 5'-terminal ribonucleotides, or at least two 3'-terminal and at least two 5' terminal ribonucleotides, and the ribonucleotides are 2'-substituted ribonucleotides. Various methods are also claimed.

Applicant does not necessarily agree with the characterization of the cited references in the instant Office Action or that the cited references provide all of the teachings for which they are cited. Some of these discrepancies are discussed below.

Furthermore, as discussed in the Amendment and Request for Continued Examination Pursuant to 37 C.F.R. § 1.114 dated September 22, 2003, M.P.E.P. § 716.01(a) states that

> [t]he Court of Appeals for the Federal Circuit stated in *Stratoflex, Inc. v. Aeroquip Corp.*, . . . that "evidence rising out of the so-called 'secondary considerations' must always when present be considered en route to a determination of obviousness." Such evidence might give light to circumstances surrounding the origin of the subject matter sought to be patented. As indicia of obviousness or unobviousness, such evidence may have relevancy.

(citations omitted) M.P.E.P. § 716.02 further states that "[a]ny differences between the claimed invention and the prior art may be expected to result in some differences in properties. The issue is whether the properties differ to such an extent that the difference is really unexpected." As stated in M.P.E.P. § 716.02(a), "[e]vidence of unobvious or unexpected advantageous properties, such as superiority in a property the claimed compound shares with the prior art, can rebut *prima facie* obviousness" and "[a]bsence of property which a claimed invention would have been expected to possess based on the teachings of the prior art is evidence of unobviousness." (M.P.E. P. 700-259)

Work published after the filing of the priority application in this case indicates that the oligonucleotides of the claimed embodiments of the present invention produced unexpected results. As discussed in Agrawal (*Biochimica et Biophysica Acta* 1489:53-68 (1999), previously submitted and attached hereto as Exhibit D), the presence of a CpG motif in phosphorothioate oligonucleotides adds to the severity of the toxicity of the oligonucleotide. (Agrawal, page 58) The sequence to which the claimed oligonucleotides are complementary, nucleotides 324 to 345 of a conserved *gag* region of the HIV-1 genome, contains one CpG motif. However, this paper teaches that incorporation of 2'-O-methylribonucleosides at both the 3'- and 5'-ends of an oligonucleotide complementary to the *gag* gene of HIV-1, in which the CpG motif was

also modified, produced an oligonucleotide which showed reduced toxicity. (Agrawal, page 60) Furthermore, incorporation of 2'-O-methylribonucleosides at both the 3'- and 5'-ends of an oligonucleotide complementary to the *gag* gene of HIV-1, in which the CpG motif was not modified, produced an increase in toxicity. (Agrawal, page 60)

As a result of the positioning of the nucleotide sequence to which the specific 21mers of the claimed embodiments of the present invention are complementary, any modification to at least two nucleotides at the 5' end of the oligonucleotide, such as having 2'-substituted ribonucleotides at the 5' end, will modify at least the C of the CpG sequence. Thus, as a result of the specific sequence to which the antisense oligonucleotide is complementary and the modifications thereto, the oligonucleotides of the claimed invention have decreased toxicity, which is an unexpected result, given the presence of a CpG motif in the nucleotide sequence.

Furthermore, as discussed above, Agrawal *et al.* does not teach or suggest any motivation for selecting the particular 21mer nucleotide sequences of the claimed invention.

One of skill in the art would not be motivated to combine the primary reference (Agrawal *et al.*) with Goodchild *et al.* or Hovanessian *et al.* to achieve the claimed invention.

Applicant previously stated that "Goodchild *et al.* refers to the use of the initiator codon for the *gag* gene as a possible sequence to which an oligonucleotide could be complementary, but does not refer to the nucleotides 324 to 345 of the *gag* gene, itself, and does not suggest using the *gag* gene, itself, as such a sequence." While this statement is generally accurate, Applicant notes that nucleotides 324 to 345 of the *gag* gene actually include the initiator codon for the *gag* gene. However, Applicant still submits that Goodchild *et al.* does not cure the deficiencies of Agrawal *et al.* As discussed above, as a result of the specific sequence to which the antisense oligonucleotide is complementary and the modifications thereto, the oligonucleotides of

the claimed invention have decreased toxicity, which is an unexpected result, given the presence of a CpG motif in the nucleotide sequence. Goodchild *et al.* does not teach or suggest either using the particular 21mer nucleotide sequences of the claimed embodiments of the invention or the unexpected result that the oligonucleotides of the claimed invention have decreased toxicity given the presence of a CpG motif in the nucleotide sequence. Therefore, Goodchild *et al.*does not cure the deficiencies of Agrawal *et al.*

Hovanessian *et al.* relates to transmembrane envelope proteins of HIV-2. Hovanessian *et al.* does not teach or suggest antisense oligonucleotides. Furthermore, Applicant submits that this reference teaches away from the alleged proposition that there is homology between HIV-1 and HIV-2 nucleic acids that would suggest an expectation of success, by emphasizing the differences between HIV-1 and HIV-2, rather than their similarities. For example, this reference states that HIV-2 and SIV-mac share about 75% overall nucleotide sequence homology, but both of them are only distantly related to HIV-1 with about 40% overall homology (col. 1, lines 31-34).

Therefore, one of skill in the art would not be motivated to combine these references with the '721 patent.

Furthermore, as discussed above, neither Hovanessian *et al.* nor Goodchild *et al.* cures the deficiency of Agrawal *et al.* recited above with regard to the rejected claims. Therefore, even if combined, Agrawal *et al.* in view of Hovanessian *et al.* and Goodchild *et al.* does not teach all of the limitations of the claims.

Likewise, the same arguments are applicable to the other independent claim (claim 16) as well as the dependent claims (claims 4, 5-11, 14-15, 18-26, and 29-39).

Accordingly, Applicant respectfully requests that this rejection be reconsidered and withdrawn.

CONCLUSIONS

In view of the arguments set forth above, Applicant respectfully requests reconsideration and reexamination of the above-referenced patent application. Applicant submits that the rejections contained in the Office Action mailed on June 16, 2004, have been overcome, and that the claims are in condition for allowance.

Applicant encloses herewith a Petition for a One Month Extension of Time pursuant to 37 C.F.R. § 1.136, until October 18, 2004 (October 16, 2005 being a Saturday and October 17, 2004 being a Sunday), to respond to the Examiner's Office Action mailed on June 16, 2004. Please charge our Deposit Account No. 08-0219 the $55.00 fee for this purpose.

Applicant also encloses herewith a Supplemental Information Disclosure Statement. Please charge the $180.00 fee for this submission to Deposit Account No. 08-0219.

No other fees are believed to be due in connection with this response. However, please charge any underpayments or credit any overpayments to Deposit Account No. 08-0219.

If the Examiner believes that any further discussion of this communication would be helpful, please contact the undersigned at the telephone number provided below.

Respectfully submitted,

Ann-Louise Kerner, Ph.D.
Reg. No. 33,523

DATE: October 18, 2004

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Exhibit A

Contact: Kristina Peterson
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PHASE II STUDY OF ANTISENSE DRUG ISIS 2302 DEMONSTRATES SIGNIFICANT AND LONG-LASTING IMPROVEMENT OF SYMPTOMS IN PATIENTS WITH ULCERATIVE COLITIS

Carlsbad, CA, October 10, 2001 – Data from a Phase II clinical trial demonstrated that the antisense drug ISIS 2302 improved symptoms of patients with active distal ulcerative colitis (UC). Patients receiving an enema formulation of ISIS 2302 experienced a dose-dependent reduction in disease activity index score (DAI) and clinical activity index score (CAI). In the trial, the median percent reduction in DAI score at the highest dose studied was highly statistically significant compared to placebo at the end of one month of dosing ($p=0.004$) and two months following cessation of dosing ($p=0.04$). Dr. S. J. H. van Deventer, Academic Medical Center, Amsterdam, Department of Gastroenterology and Hepatology, presented these study results today at the United European Gastroenterology meeting in Amsterdam. IsisPharmaceuticals, Inc. (NASDAQ: ISIP), is developing ISIS 2302, an antisense inhibitor of intercellular adhesion molecule-1 (ICAM-1), a molecule that plays a central role in inflammation.

"Based on this first clinical experience, the retention enema formulation of ISIS 2302 appears promising as a potential new treatment for ulcerative colitis," said Dr. S. J. H. van Deventer. "We observed significant activity at the higher doses studied, and the drug and method of administration were well tolerated by patients."

The Phase II randomized, placebo-controlled dose-escalation study involved 40 people with active distal UC at 11 European trial sites. Patients participating in the study had UC for an average of six years and were allowed to remain on a standard oral medication for the disease. Patients were randomized to receive 60 mL enemas containing placebo, 0.1, 0.5, 2 or 4 mg/mL of ISIS 2302 every night for one month. Patients were evaluated for improvements in DAI and CAI scores upon completion of dosing and during the five-month follow-up period.

Results showed that at the end of one month of dosing, patients who received a 4 mg/mL dose of ISIS 2302 experienced a median improvement in DAI score of 73%, compared to a 23% improvement for placebo patients ($p=0.004$). The median CAI score of these patients improved as well, 58% for drug-treated patients versus 32% for placebo. These median improvements in DAI and CAI scores (73% and 58%, respectively) were maintained at month three and month six of the study. Two patients in this dosing group experienced full endoscopic remission at the end of the one month dosing period. No patients in the 4 mg/mL group required additional medications during the entire six month trial period, while 50% of the placebo patients required new medications to control their symptoms.

-more-

"We are very encouraged by the favorable results we've observed in this trial of ISIS 2302 in ulcerative colitis, particularly with the durability of response after a single month of dosing," said F. Andrew Dorr, M.D., Isis' Vice President and Chief Medical Officer. "These patients currently have few alternatives to surgery to control their disease. We will continue to refine the dosing schedule and evaluate the market potential of this new product opportunity."

ISIS 2302 is an antisense inhibitor of ICAM-1, a molecule that plays a key role in a wide range of inflammatory and autoimmune conditions such as UC. ICAM-1 is part of a family of molecules, the cellular adhesion molecules, that can be found on the surface of virtually every cell in the body, including cells that line the colon. It is involved in the production of immune factors that cause the inflammatory response in UC. ISIS 2302 is also being studied in Crohn's disease and a topical formulation for psoriasis.

According to the Crohn's and Colitis Foundation of America, UC is an inflammatory disease of the colon, the large intestine, which is characterized by inflammation and ulceration of the innermost lining of the colon. Symptoms characteristically include diarrhea, rectal bleeding and abdominal pain. UC differs from another inflammatory bowel disease, Crohn's disease, as it only affects the colon. An estimated 500,000 Americans have UC.

Isis will conduct a live webcast conference call to discuss this release on Wednesday, October 10 at 10:30 AM Eastern time. To participate over the internet, go to www.isip.com. A replay of the webcast will be available at this address for up to 90 days.

Isis Pharmaceuticals, Inc. is exploiting its expertise in RNA to discover and develop novel human therapeutic drugs. Isis has 12 antisense products in its development pipeline with two in late-stage development and six in Phase II human clinical trials. Isis' GeneTrove™ division uses antisense to assist pharmaceutical industry partners in validating and prioritizing potential gene targets through customized services and access to an extensive gene function database. Ibis Therapeutics™ is a division focused on the discovery of small molecule drugs that bind to RNA. Isis has a broad patent estate as the owner or exclusive licensee of more than 800 issued patents worldwide.

This press release contains forward-looking statements concerning the clinical development of ISIS 2302, its prospects as a treatment for ulcerative colitis and the potential of Isis' drug discovery program. Such statements are subject to certain risks and uncertainties, particularly those inherent in the process of discovering, developing and commercializing drugs that are safe and effective for use as human therapeutics and financing such activities. Actual results could differ materially from those projected in this release. As a result, the reader is cautioned not to rely on these forward-looking statements. These and other risks concerning Isis' research and development programs are described in additional detail in the Registration Statement on Form S-3 filed October 9, 2001 with the U.S. Securities and Exchange Commission, copies of which are available from the company.

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Exhibit B

Drug Development

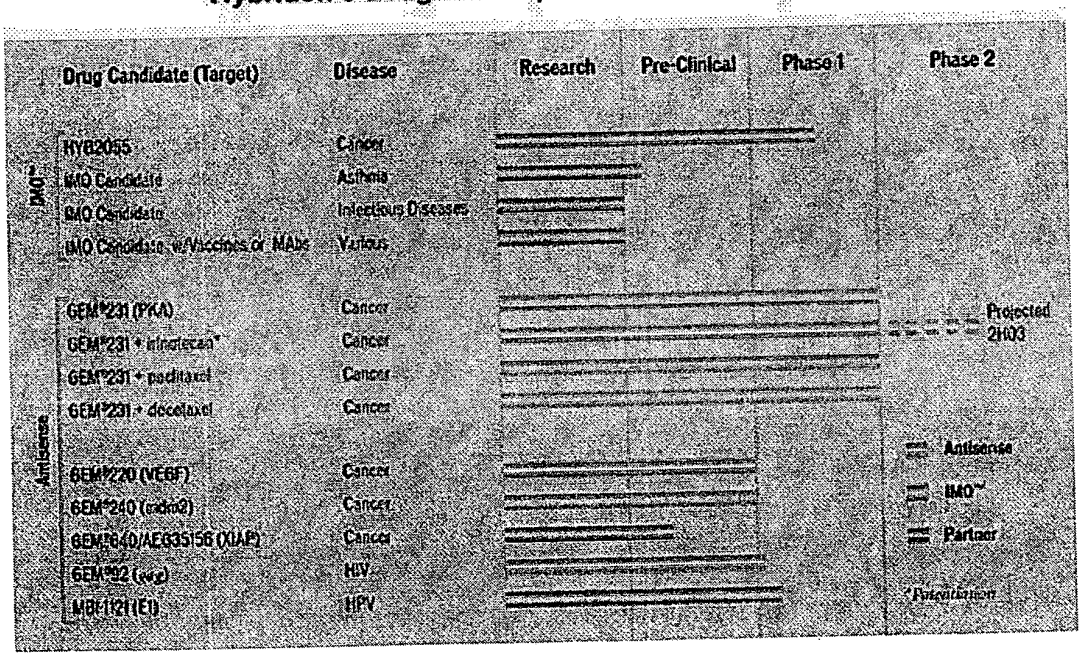
Clinical programs

As a pioneering company in antisense technology, Hybridon has developed extensive experience, both in preclinical and clinical studies, involving the systemic administration of antisense drug candidates. Results of early trials in man indicated that first generation antisense agents produced certain side effects that were not anticipated from preclinical studies. These results prompted Hybridon further to modify chemically its antisense agents, leading to the identification of a series of 2nd generation antisense agents with improved pharmaceutical properties. These advanced chemistry antisense agents have increased metabolic stability (providing the potential for administration by the oral route), enhanced potency, and an improved side effect profile.

All of Hybridon's antisense drug candidates are 2nd generation, antisense agents. Hybridon's initial product focus is in the therapeutic areas of oncology and viral disease.

In the area of immune modulation, Hybridon has begun the clinical development of its lead IMO™ compound, HYB2055. This drug candidate is a synthetic compound with advanced chemical modifications which Hybridon believes may offer advantages over earlier CpG oligos. Based on Hybridon's expertise in synthetic DNA chemistry and an understanding of the structure-activity relationship by which IMO™ compounds can modulate the vertebrate immune system, HYB2055 is only the first in what Hybridon expects to be a series of IMO™ compounds, each of which can be fine-tuned with to desired potency, specificity and duration of action.

Hybridon's Drug Development Product Pipeline



Hybridon has three drug candidates in the clinical phase of development.

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conducted at Vanderbilt University dical Center and the University of Chicag edical Center. Hybridon plans to complete this trial in 2003.

GEM®231 is a 2nd generation antisense compound for treating solid tumor cancers. We chose to evaluate the combination of GEM®231 and Camptosar based on promising preclinical data relating to this combination as a treatment of solid tumor cancers.

In the clinical trial, we are evaluating the safety of GEM231 and Camptosar in combination and measuring the presence of extra-cellular PKA, or ECPKA, in blood as a biomarker for GEM231 antisense activity. A biomarker is a biological parameter monitored as a possible indicator of drug activity.

GEM®231 is designed to inhibit protein kinase A, or PKA. PKA is a protein that plays a key role in the control of the growth and differentiation of mammalian cells. Levels of PKA have been shown to be increased in the cells of many human cancers, and high levels of PKA have been shown to correlate with unfavorable clinical outcomes in patients with breast, colon and ovarian cancers.

We previously conducted other phase 1/2 clinical trials of GEM®231, both as a monotherapy and in combination with other marketed chemotherapeutics. We believe that these trials involved the first systemic administration of a 2nd generation antisense compound to oncology patients. In December 2002, we completed a phase 1/2 study of 14 patients with solid tumors undergoing treatment with GEM®231 as a monotherapy. In the study, ECPKA was monitored for each patient before and during the treatment as a biomarker for GEM®231 activity. Results of the study showed a decrease in ECPKA in patients.

In the phase 1/2 trial of GEM®231 as a monotherapy and in our other trials of GEM®231, we also evaluated the safety of GEM®231 in multiple doses in oncology patients. These trials explored the maximum tolerated dose of GEM®231 for both single doses and multiple doses. In the trials, GEM®231 was generally well tolerated. Even in high doses, GEM®231 did not show some of the side effects normally associated with most current cancer treatments or with 1st generation antisense compounds.

GEM®92

Status: The initial single dose phase 1 study of GEM®92 orally at three dose levels and by injection showed excellent safety results and successfully confirmed in humans oral delivery of 2nd generation antisense agents. Hybridon is evaluating the market opportunity for GEM®92 in view of the current effectiveness of HAART therapies in treating HIV. We may recommence our development efforts if and when viral resistance to existing therapies results in a need for a new approach to HIV treatment.

Based on the clinical experience we gained with GEM®91, our 1st generation antisense compound that also targeted the same region of HIV-1, we created chemical modifications to improve the side effects profile and to enhance the stability of the compound. In 1997, we conducted a phase 1 study in the United Kingdom to investigate the safety and pharmacokinetics of single doses of GEM®92, given at three different dose levels by the oral route and one does level as a 2-hour intravenous infusion. All doses given in the study were well tolerated by the patients. Further, GEM®92 was detected in the blood after both oral dosing and injection, suggesting that GEM®92 could be developed as an oral drug. We believe both that the medicinal approach and genetic target for GEM®92 are unique in that no antisense drug has been approved for the treatment of AIDS, and no other drug has the same target on the HIV-1 genome.

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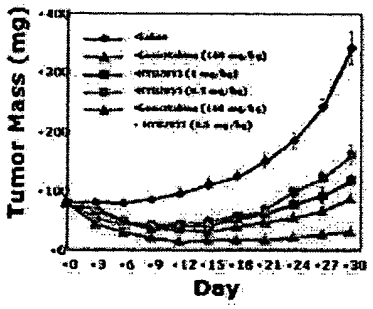
Immunomodulatory Oligonucleotides (IMO™)

HYB2055

Anti-Tumor* / ivity of HYB2055



* Human prostate cancer PC-3 in male nude mice; IMO™ Treatment Day 1, 3, 5/week

immunologic activity and pharmacokinetics of HYB2055 in healthy individuals. In May 2003, a phase 1 clinical trial was initiated at Georgetown University's Lombardi Cancer Center in cancer patients with malignant solid tumors.

HYB2055 is the lead compound in Hybridon's IMO™ program. HYB2055 is an immune stimulator. Preclinical data in animal and human cells has shown HYB2055 activates cells of the immune system to secrete a number of effector molecules, or cytokines. In animals bearing various human cancer xenographs, HYB2055 shows potent anti-tumor activity.

In addition to cancer, we believe the IMO™ compounds used in HYB2055 could also have use as a monotherapy for treatment of allergic asthma and other allergies and infectious diseases. IMO™ compounds may also be useful as co-factors in combination therapeutic agents with vaccines, monoclonal antibodies (MAbs), peptides, chemotherapeutic agents, allergens and antigens.

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Collaborations

MG98

Status: Phase 1 dose-escalation clinical trials of MG98 have been completed. Phase 2 clinical trials have been initiated. For further updates, visit www.methylgene.com.

Work on antisense inhibitors of DNA methyl transferase began as a Hybridon research project, in collaboration with Dr. Moshe Szyf of McGill University. The enzyme, DNA methyltransferase, is considered to be a key regulator responsible for maintaining aberrant DNA methylation in human cancer cells. The first drug candidate developed by MethylGene to enter clinical development is MG98, a 2nd generation antisense agent that inhibits the production of DNA methyl transferase.

MethylGene Inc was formed in 1996 as a Hybridon spinout company. In April 2001, Hybridon sold its equity interest in MethylGene.

MBI 1121 (FORMERLY ORI 1001)

Status: MBI 1121 is currently in phase 1. For further updates, visit www.mbiotech.com.

MBI1121 is a 2nd generation antisense analog that targets the E1 protein of human papillomavirus (HPV). The E1 protein is a highly conserved element across a number of HPV types and has an essential role in viral replication. In September 2002, Micrologix acquired ORI 1001, which was formerly owned, and being developed by Origenix Technologies Inc. for the treatment of diseases associated with (HPV) such as external genital warts. In conjunction with the acquisition of this HPV molecule, Micrologix entered into a collaboration with Hybridon for exclusive rights for a patent covering HPV antisense oligonucleotides for use against the HPV genome as well as the non-exclusive rights to a portfolio of antisense oligonucleotide chemistry patents.

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Exhibit C

Oligonucleotides

First-generation HIV-Antisense Oligonucleotide Trials Halted

On Friday, July 28, 1997, Hybridon Inc., of Cambridge, Massachusetts, announced that is halting development of GEM91, its first-generation antisense oligonucleotide directed against the HIV gag gene, because of platelet depletion in trial patients. The company will focus efforts on GEM92, a second-generation antisense compound, which also targets the gag site of the viral genome.

Earlier trials indicated that GEM91 significantly reduced viremia in HIV-positive patients treated for up to eight days. However, in the latest Phase IIb trial, 3 of 9 patients with advanced HIV infection experienced a decrease in platelet count after ten days of treatment.

This finding led to the decision to halt development of the compound. In a company communication, Hybridon VP Dr. Russell Martin said, "...even if efficacy could be demonstrated, the results of this Phase II clinical trial indicated that chronic therapy for advanced HIV patients with GEM91 in combination with other antiretrovirals likely would require periodic interruption of drug administration."

The second-generation antisense products being developed by Hybridon, unlike the first-generation phosphorothioates, are mixed backbone oligonucleotides. "We believe these second generation oligonucleotides will have a substantially greater therapeutic index," said Hybridon Chairman E. Andrews Grinstead. He added, "The growing population of AIDS patients who...develop resistance to triple combination therapy may be helped by GEM92."

GEM92 is expected to enter clinical trials in October. So far, animal studies show that it causes substantially fewer platelet and liver changes than GEM91, and that it has the potential for oral administration. Dr. Martin said he hopes GEM92 will have "...increased potency, reduced frequency of dosing administration and an improved safety profile, as compared to first generation antisense compounds."

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Review

Importance of nucleotide sequence and chemical modifications of antisense oligonucleotides

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Abstract

The antisense approach is conceptually simple and elegant; to design an inhibitor of a specific mRNA, one needs only to know the sequence of the targeted mRNA and an appropriately modified complementary oligonucleotide. Of the many analogs of oligodeoxynucleotides explored as antisense agents, phosphorothioate analogs have been studied the most extensively. The use of phosphorothioate oligodeoxynucleotides as antisense agents in various studies have shown promising results. However, they have also indicated that quite often, biological effects observed could be solely or partly non-specific in nature. It is becoming clear that not all phosphorothioate oligodeoxynucleotides of varying length and base composition are the same, and important consideration should be given to maintain antisense mechanisms while identifying effective antisense oligonucleotides. In this review, I have summarized the progress made in my laboratory in understanding the specificity and mechanism of actions of phosphorothioate oligonucleotides and the rationale for designing second-generation mixed-backbone oligonucleotides.

Keywords: Antisense; Oligonucleotide; Mixed-backbone oligonucleotide; Pharmacokinetics; Immune stimulation; CpG; Pro-drug; Antiviral

Contents

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1. Introduction

Antisense oligonucleotides provide a rationally designed tool to manipulate expression of specific gene products [1,2]. In the last 10 years, antisense oligonucleotides have been widely used in various in vitro and in vivo models [3–11]. Based on the promising results obtained, antisense oligonucleotides are being explored for their potential as therapeutic agents for the treatment of viral infections, cancers and inflammatory disorders [5,6,8,9]. The antisense approach is conceptually simple; to design an inhibitor, one needs only to know the sequence of the targeted gene product (mRNA) and to identify any specific modifications of the oligonucleotide. Antisense oligonucleotides have been used extensively in the last several years, with mixed results. [8,10–12]. The most-studied of the oligodeoxynucleotides are the phosphorothioate analogs (PS-oligos), in which one of the non-bridging oxygen atoms in the phosphate backbone is replaced by a sulfur [3–11]. Based on the results obtained to date with PS-oligos, it has become evident that PS-oligos exert biological activity by multiple mechanisms of actions [8–13]. The mechanisms of action can be classified into three categories: (a) sequence-specific activity by binding to mRNA, referred here as antisense activity; (b) sequence-specific activity by interacting with other factors than mRNA, referred here as non-antisense activity; and (c) non-sequence-specific activity. In this review, I have attempted to summarize the progress in my laboratory in understanding the rules that govern the specificity and mechanisms of actions of PS-oligos and the rationale for designing second-generation antisense oligonucleotides. A number of other investigators have published their results and views, which also appear in this issue.

2. Phosphorothioate oligodeoxynucleotides

PS-oligos have a negatively charged backbone and are capable of supporting RNase-H activity similar to phosphodiester oligodeoxynucleotides, but PS-oligos have greater resistance to nuclease degradation than do phosphodiesters. These intrinsic properties have made PS-oligos the choice as first-generation antisense oligonucleotides [3–11].

We and others initially used PS-oligos as inhibitors of HIV-1 replication in HIV-1-infected cells [14–23]. From the results obtained it was obvious that the PS-oligos effectively inhibited HIV-1 replication, but the apparent mechanism of HIV-1 inhibition differed depending on the experimental model [14–22]. PS-Oligos inhibited HIV-1 replication by antisense mechanisms as well as non-sequence-specific mechanisms [14–25]. The non-sequence-specific mechanism was most probably due to the polyanionic nature of PS-

oligos [14–25]. Similar results were observed with PS-oligos used to inhibit replication of influenza virus and other viruses [26]. In some recent studies, the biological activity observed with PS-oligos has also been associated with their polyanionic nature [27,28].

In the last six years, there have been many reports in which PS-oligos have been used to inhibit overexpression of cellular gene products implicated in cancer and inflammation. The results strongly suggest that the inhibition observed in these studies was primarily due to antisense activity [3–11,30–36]. We recently used PS-oligos complementary to an MDM2 oncogene [37]. The MDM2 oncogene encodes for an inhibitor of the p53 tumor suppressor protein that regulates p53 in a negative feedback loop [38,39]. In this study, selected antisense PS-oligos inhibited MDM2 expression at both the mRNA and protein levels [37]. Suppression of MDM2 oncoprotein led to a decrease in MDM2-p53 complex formation, which in turn resulted in an increase in p53 transcriptional activity, and finally to apoptosis [37]. The effects observed were sequence specific, as the control PS-oligos (with four mismatches) did not show such activity.

It has been shown in a number of studies that PS-oligos with CpG motifs have immune-stimulatory properties in rodents [11,40–46]. The severity of immune stimulation depends on the position of the CpG motif and its flanking sequence of PS-oligos [40–46]. PS-Oligos containing CpG motifs are known to induce cytokines, including IL-6, IL-12, TNF-α, gamma-IFN [43,44], and also chemokines [45]. These cytokines induced by PS-oligos containing CpG motifs have been shown directly or indirectly to have antiviral [47,48], anticancer [49], and antibacterial activities [50].

3. Pharmacokinetics and tissue distribution

Pharmacokinetics of PS-oligos in mice following intravenous administration showed rapid elimination from the plasma compartment with half-lives ranging from 30 min to 1 h [51]. PS-Oligo was distributed to highly perfused organs, such as kidney, liver, bone marrow, and spleen, in higher concentration than other tissues [51]. The primary route of elimination was in urine, with smaller amounts found in feces [51]. Following intraperitoneal or subcutaneous administration, no significant differences in tissue distribution were observed, except that lower maximum plasma concentrations were achieved than with intravenous administration [51,52]. The analysis of extracted PS-oligos from plasma and tissues showed the presence of both intact and degraded forms of the PS-oligo [51,52]. Protection of PS-oligos on the 3′-end significantly minimized degradation, this suggested that in vivo degradation was primarily due to 3′-exo-nucleases [53]. Detailed analysis of the extracted oligo showed that the PS-oligo was degraded primarily from the 3′-end, but some degradation products were generated following degradation from the 5′-end and from both the 3′- and 5′-ends [54]. Similar pharmacokinetic and tissue disposition results were obtained in rats [55] and monkeys [56]. In humans, the plasma pharmacokinetic profile and elimination in urine were similar to those observed in monkeys [57]. Similar results, in general, have been reported with PS-oligos of varying sequences [58]. The pharmacokinetics of PS-oligos are found to be largely sequence independent except for PS-oligos that can form hyperstructures (e.g., G-rich oligos) [59].

4. Safety

Safety studies of PS-oligos in mice and rats show sequence-dependent side effects [10,11]. These side effects include splenomegaly, thrombocytopenia, and elevation of the liver enzymes, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) [10,11,60]. Histopathological examinations have revealed multi-organ mononuclear cells infiltrates, reticuloendothelial cell and lymphoid hyperplasia, and renal tubule degeneration [60]. The severity of these side effects are dependent on the dose and the frequency and duration of administration [60]. Similar results have been reported with other PS-oligos [61].

The safety profile of PS-oligos in monkeys is different from that observed in mice and rats [62]. Intravenous administration of PS-oligos in monkeys caused a brief increase, followed by a prolonged decrease, in arterial blood pressure, and a transient decrease in peripheral total white blood cells and

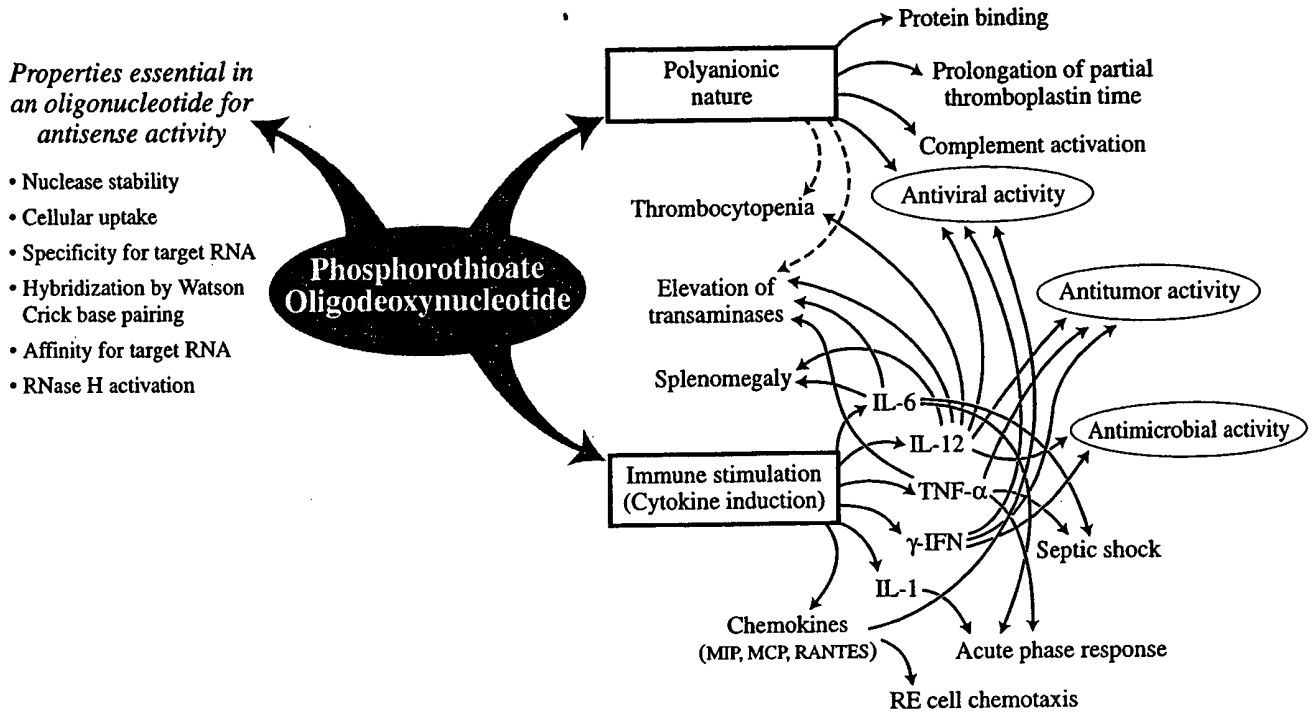

Fig. 1. Factors affecting the mechanism of action of oligonucleotides. Antisense oligonucleotides are designed to bind to targeted mRNA and inhibit translation. An effective oligonucleotide should be stable to nucleases and taken up by cells. In addition, oligonucleotides should have strong affinity for the target mRNA and should activate RNase H for RNA cleavage. Based on the nucleotide sequence and the nature of the internucleotide linkages, however, oligonucleotides display additional properties that may interfere with the specificity and mechanism of action of the oligonucleotide. Two major properties are the polyanionic nature and immune stimulation. As shown above in a generalized way, the polyanionic nature and immune stimulation may produce biological activities and side effects that compromise the specificity and action of a given oligonucleotide. Minimization of polyanionic nature and immune stimulatory properties by appropriate chemical modification should be considered in second-generation oligonucleotides.

neutrophil counts [62]. In addition, complement activation and prolongation of aPTT have also been observed [62]. These side effects are sequence independent, however, dependent on the concentration of PS-oligo in the plasma compartment, and can be minimized by slow intravenous infusion [62]. A similar observation has been made with other PS-oligos [63]. PS-Oligos that can form hyperstructures (e.g., G-rich oligos) have greater effects on complement activation and prolongation of aPTT [64].

A PS-oligo (GEM 91) has been administered to humans by 2- or 24-h intravenous infusion. The side effects observed included thrombocytopenia, elevation of transaminases, and prolongation of aPPT [65]. These side effects were dependent on the dose and duration of the treatment and the frequency of administration. Administration of lower doses of PS-oligos had minimal changes in the above mentioned side effects [65–67].

5. Understanding of PS-oligos

On the basis of knowledge gained to date with PS-oligos, the following generalizations can be made.

1. PS-oligos have built-in properties (e.g., affinity for target mRNA, nuclease stability, cellular uptake, and RNase H activation) required for antisense activity (Fig. 1).
2. PS-oligos behave like polyanionic molecules as is evident from the inhibitory activity observed in viral replication assays, binding to growth factors, serum proteins, etc. In addition, activation of complement and prolongation of aPTT in monkeys and humans is associated with the polyanionic characteristics of PS-oligos (Fig. 1).
3. PS-oligos have sequence-specific biological activities, which are not due to hybridization to specific mRNA. Two types of mechanisms may be in-

volved: immune stimulation (Fig. 1) and binding of factors (e.g., transcription factor and proteins) to a decoy or specific motif of PS-oligos [67–69].

6. Beyond PS-oligos

The use of PS-oligos in various studies has shown that antisense oligonucleotides can be used to selectively inhibit the expression of targeted mRNA in cell culture and in vivo [3–11,29–36]. Certain undesirable properties (e.g., polyanionic nature, immune stimulation) limit their potential for wider use as therapeutic agents and also as tools in elucidating the function of specific target genes (Fig. 1).

To overcome some of the limitations of PS-oligos, extensive efforts have been made to synthesize various analogs of oligonucleotides [3–11,70]. These analogs include deoxynucleotides with modification of the internucleotide linkages, heterocyclic bases, or sugar [3–11,70]. Some of these analogs have higher affinity for target RNA and increased resistance to serum and cellular nucleases than PS-oligos. Studies of these analogs in various cell culture models have not yielded encouraging results compared with those obtained with PS-oligos.

In the last few years, we have made attempts to rationally minimize the undesirable properties of PS-oligos, while maintaining the properties essential for antisense activity (Fig. 1). The following are two examples in this respect.

7. Minimization of polyanionic nature of PS-oligos

The polyanionic characteristics of PS-oligos have been associated with some of the observed non-sequence-specific biological activities [14,15,21,23,25–28] and also with complement activation and prolongation of aPTT [62,71,72]. In our studies using phosphorothioate oligoribonucleotides, phosphorothioate 2′-*O*-methyloligoribonucleotides, and phosphorothioate 2′,5′-oligoribonucleotides, it became clear that the polyanionic nature of these analogs is somewhat different from that observed with PS-oligos [73]. These analogs had less of an effect on complement activation and prolongation of aPTT, suggesting that not only the phosphorothioate linkage, but also the nature of the nucleosides of PS-oligos, is responsible for the polyanionic characteristics. It is possible that Rp and Sp diastereomers of PS-oligos have different polyanionic characteristics; one of the isomers of PS-oligoribonucleotides has less pronounced polyanionic characteristics because of the 2′-hydroxyl group or other 2′-modifications.

There have been no reports on the polyanionic characteristics of Rp and Sp stereospecific PS-oligos. We have observed that Rp stereospecific PS-oligos have a greater affinity for complementary RNA and less stability towards nucleases than do stereo-random PS-oligos [74]. Also, Rp PS-oligos were better substrates for RNase H than were stereo-random PS-oligos [74]. Independent studies using Sp stereospecific PS-oligos have shown that they have less of an affinity for complementary RNA and greater stability towards nucleases than do Rp and stereo-random PS-oligos [75]. Lack of efficient synthetic methodologies to obtain stereospecific Rp or Sp PS-oligos have been preventing us from performing detailed studies. Recently, however, we have succeeded in synthesizing stereo-enriched Rp and Sp PS-oligos and also PS-oligos with an appropriate mix of Rp and Sp linkages [76,77]; detailed studies are now under way.

To minimize the polyanionic nature of the PS-oligos, we have taken advantage of the reduced polyanionic characteristics of 2′-*O*-methylribonucleosides, (as observed by reduced complement activation and prolongation of aPTT), and have substituted a few deoxynucleosides with 2′-*O*-methylribonucleosides, either at the 3′-end or both the 3′- and 5′-ends or in the center of the PS-oligos [78–80] (Fig. 2). The overall result of this substitution is increased affinity to target RNA, stability towards nucleases, and reduced polyanionic-related side effects. In addition, these oligonucleotides retain the RNase H activating capability due to the presence of PS-oligo [78–80]. Similar results have been obtained by incorporating other modified oligonucleotides [81–83]. In addition to chemical modifications, polyanionic related effects can be minimized with the use of appropriate formulations. Use of protamine has been shown to minimize these effects [72].

Table 1
Structure and sequence of oligonucleotides

1.	TCG TCG CTG TCT CCG CTT CTT CTT GCC
2.	$T^{me}CG$ $T^{me}CG$ CTG TCT $C^{me}CG$ CTT CTT CTT GCC
3.	TC_mG TC_mG CTG TCT CC_mG CTT CTT CTT GCC
4.	[TCG] [TCG] CTG TCT C[CG] CTT CTT CTT GCC
5.	CTC TCG CAC CCA TCT CTC TCC TTC T
6.	CTC TC_mG CAC CCA TCT CTC TCC TTC T
7.	CTC TGC CAC CCA TCT CTC TCC TTC T
8.	CTC $T^{me}CG$ CAC CCA TCT CTC TCC TTC T
9.	[CG C]AC CCA TCT CTC TCC [UUC U]
10.	GCG TGC CTC CTC ACT GGC
11.	CGC CGG GAT CTC GAT GCT CAT
12.	CCG CTC TTC CTC ACT GGT
13.	[GCG U]GC CTC CTC AC[U GGC]
14.	CTC TCG CAC CCA TCT CTC TCC TTC T (m m m m m m m m m)
15.	CTC TCG CA[C CCA UCU C]TC TCC TTC T
16.	CTC TCG CA[C CCA UCU C]TC TCC TTC T

All sequences are phosphorothioate; C^{me} - 5-methyl cytosine;
X_mX - methylphosphonate linkage; [XX] - 2'-O-methyl ribonucleoside;
[XX] - 2'-O-methylribonucleoside with phosphodiester linkages.
Oligo 1 is complementary to *rev* gene of HIV-1; oligo 5 is complementary to *gag* gene of HIV-1; oligo 10 is complementary to R1α subunit of human protein kinase A (PKA); oligo 11 is complementary to RIIβ subunit of human PKA; oligo 12 is complementary to R1α subunit of mouse PKA.

8. Minimization of immune stimulation by PS-oligos

PS-oligos containing a CpG motif and the appropriate flanking sequences are known to be immune stimulatory [11,40–46]. Recent studies have indicated that activation of immune stimulation is through induction of mitogen-activated protein kinases [84,85]. The immune stimulation by CpG-containing PS-oligos results in induction of various cytokines, which has a therapeutic effect; these PS-oligos are being developed as novel therapeutic agents [47–50]. At the same time, these cytokines at higher doses have been associated to side effects [45].

We have studied PS-oligos for their safety profile in mice and rats and have reached the conclusion that the presence of CpG motif in PS-oligos adds to the severity of the toxicity observed [11]. For example, oligo **1** (Table 1) administered to mice causes thrombocytopenia, elevation of transaminases, and enlargement of the spleen (Fig. 3A). In addition, histopathological changes in the kidney, liver, and spleen were also noted. Modification of the CpG motif significantly minimized the side effects observed with oligo **1**; these modifications included: replacement of the cytosine of the CpG motif with a 5'-methyl cytosine (oligo **2**, Table 1); replacement of the phosphorothioate linkage of the CpG motif with a methylphosphonate linkage (oligo **3**, Table 1); and replacement of the entire CpG motif with 2'-*O*-methylribonucleosides (oligo 4, Table 1) (Fig. 3A). Minimization of the histopathological changes was also noted with oligos **2**, **3**, and **4**. These modifications suppressed the immune stimulatory properties of the CpG motif [42]. Reduction in the toxicity of oligo **1** produced by these modifications strongly suggests that PS-oligos have similar toxicities, but the severity of toxicity is increased due to presence of CpG motif [11] and their immune-stimulatory properties. Similar results were observed in rats with oligos **5**, **6**, **7**, and **8** (Fig. 3B).

It is important to note that the flanking sequence of the CpG motif is a major factor in inducing immune stimulation, and not all PS-oligos with the CpG motif will behave in the same manner [11,41]. Oligos **10**, **11**, and **12**, which contain the CpG motif at different positions in their sequences and flanking sequences, had significantly different toxicity profiles in mice (Fig. 3C). Oligo **10** showed more toxicity than oligo **11** and **12**. It is also important to note that immune stimulation due to a given PS-oligo sequence depends on the host, the dose, and the route of administration (Q. Zhao, S. Agrawal, unpublished data).

The above discussion and results suggest that a given PS-oligo can be appropriately modified to sup-

press its immune-stimulatory properties and the resulting toxicity. Furthermore, these modifications can be made more rationally to also improve general therapeutic potential of oligonucleotides as discussed below.

9. Mixed-backbone oligonucleotides (MBOs)

As it is evident from the above discussion, the nucleotide composition and nature of the nucleotide and internucleotide linkages alone or in combination dictate the biophysical, biochemical, and biological properties of oligonucleotides [70]. A number of oligonucleotides analogs have been studied that display properties different from those of PS-oligos in terms of resistance to nucleases, affinity to target RNA, cellular uptake, activation of RNase H, and more importantly, the in vivo pharmacokinetic profile [3–11]. In our earlier studies, we employed phosphorothioate oligoribonucleotides which bind to RNA with higher affinity than PS-oligos, but do not activate RNase H. They showed reduced anti-HIV activity compared to PS-oligos [17]. These results suggested that for optimum activity, antisense oligonucleotides should have combination of various properties instead of only increased stability toward nucleases or high affinity to target RNA.

We have made attempts to combine two modified oligonucleotides in order to generate a mixed-backbone oligonucleotide (MBO) that brings together the beneficial properties of the two molecules [71,72,78,80–82,86,87]. MBOs in general have two segments: one that contains an oligonucleotide analog capable of activating RNase H, and another that does not activate RNase H (Fig. 2). The oligonucleotides that activate RNase H are those bearing at least four contiguous phosphodiester or phosphorothioate

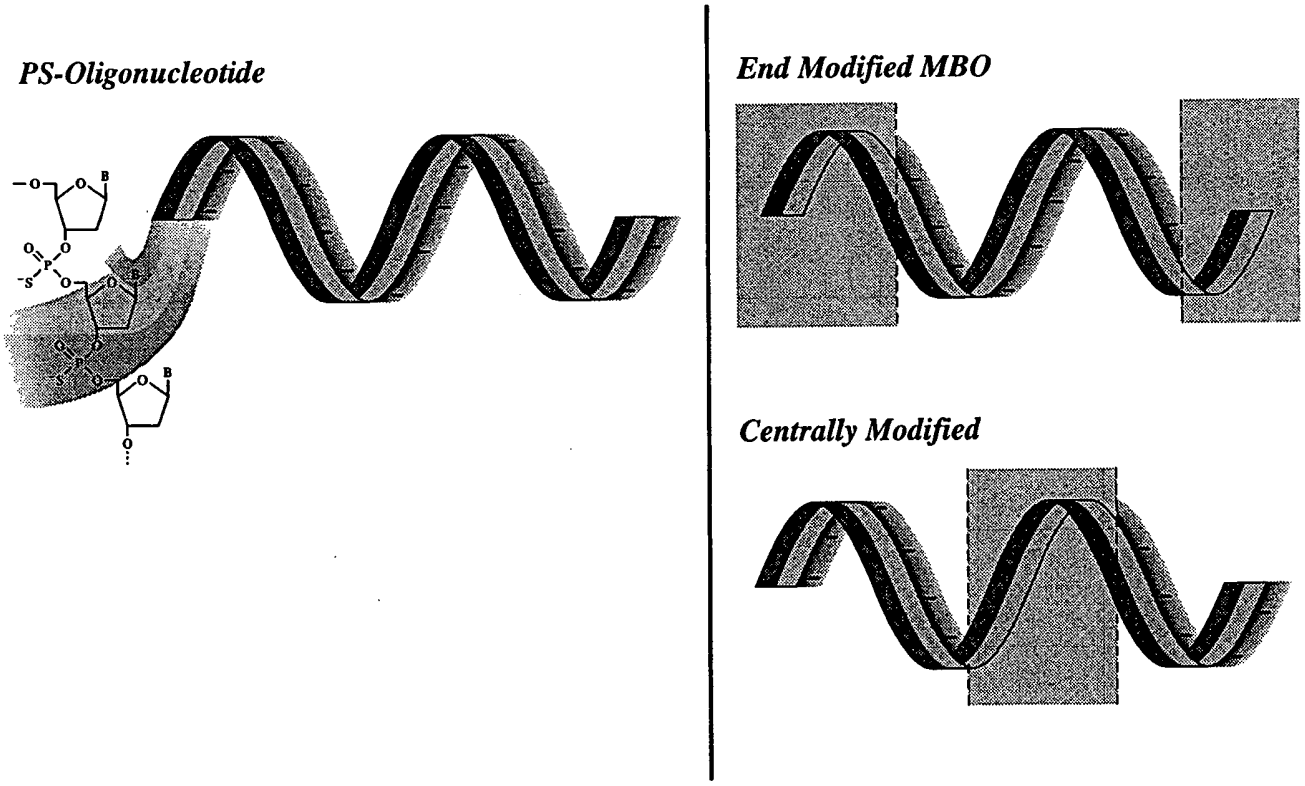

Fig. 2. Structure of PS-oligonucleotide and MBOs. In PS-oligos one of the non-bridging oxygen atoms in the phosphate backbone is substituted by a sulfur. In general, various biophysical and biochemical properties of PS-oligos are controlled by phosphate backbone, nucleoside sugar, and heterocyclic bases. To modulate many of these properties, mixed-backbone oligonucleotides have been studied. MBOs refer to oligonucleotides which combine the advantages of two different modified oligonucleotides. Based on the position of incorporation of modified oligonucleotides in PS-oligos, MBOs can be classified as end-modified MBOs and centrally modified MBOs.

internucleotide linkages [80,81]. Of the oligonucleotide analogs that do not activate RNase H, we have studied oligodeoxynucleotides containing methylphosphonate [81–83,86,87], phosphoramidate [81,88], methylthiophosphonate [89], methylphosphotriester [90], methylphosphothiotriester [90], carbamates [91], and oligoribonucleotides containing 2′-*O*-methylribonucleosides [78], 2′-*O*-methylribonucleoside with methylphosphonate linkage [92,93], and 2′-*O*-methylribonucleoside with phosphoramidate linkages (D. Yu, S. Agrawal, unpublished data), and 2′,5′-linked oligoribonucleotides [94].

The positioning of these analogs of oligonucleotides in a given sequence is crucial to the outcome and should be chosen carefully. We have studied two types of MBOs: end-modified and centrally modified MBOs.

10. End-modified MBOs

In end-modified MBOs, a non-RNase-H-activating analog of oligonucleotide is placed at the 3′-end or at both the 3′- and 5′-ends of the PS-oligo (Fig. 2). The purpose of incorporating various modified oligonucleotides in MBOs is to modulate biophysical, biochemical, or biological properties [71–73,78,80–82,86–94]. It has become evident that they also provide improvement in pharmacokinetic and safety profiles. The end-modified MBOs that have been studied extensively contain either methylphosphonate internucleotide linkages [81] or 2′-*O*-methyloligoribonucleosides [78].

End-modified MBOs, in general, have increased in vivo stability due to their increased resistance towards nucleases [78,95]. Because of this feature, some of the end-modified MBOs have shown good bioavailability following oral or colorectal administration [96,97]. The advantage of increased in vivo persistence of MBOs over PS-oligo is that it may allow less frequent administration for pharmacological activity.

Appropriate placement of the modified oligo in an end-modified MBO can decrease the toxicity of PS-oligos [98]. Oligo **5** (Table 1) administered to rats showed a toxicity profile similar to that of other PS-oligos; alteration or modification of the CpG dinucleotides reduced the toxicity (oligos **6**, **7**, and **8**, Table 1 and Fig. 3B). Incorporation of four 2′-*O*-methylribonucleosides at both the 3′- and 5′-ends of oligo **5**, in which the CpG motif was also modified, produced oligo **9**, which showed reduced toxicity (Fig. 3B). Incorporation of four 2′-*O*-methylribonucleosides at both the 3′- and 5′-ends of oligo **5**, in which the CpG motif was not modified, produced an increase in toxicity [60]. An improved safety profile was also observed with an end-modified MBO (oligo **13**) of oligo **10**. (Fig. 2) [97]. The end-modified MBOs in both cases showed similar or improved biological activity. Oligo **13** is presently in Phase I human clinical trials and have shown overall improved safety profile including complement activation, prolongation of aPTT and thrombocytopenia [99].

End-modified MBOs also produce fewer polyanionic-related side effects than do PS-oligos [71, 72]. To further minimize the polyanionic characteristics of PS-oligos, attempts have been made to reduce the number of phosphorothioate linkages in MBOs by incorporating 2′-*O*-alkylribonucleosides along with a phosphodiester backbone [79]. Incorporation of 2′-*O*-methyloligoribonucleotides containing phosphodiester linkages at both the 3′- and 5′-ends of the PS-oligo failed to provide nuclease stability comparable to that of the PS-oligo [79]. Similar results have been observed with bulkier 2′-*O*-alkyl groups, including 2′-*O*-propylribonucleosides [58]. Recently, MBOs containing 2′-*O*-methoxyethoxyribonucleosides have been studied, but there is no report of in vivo stability of these MBOs yet [100]. We recently reported that in end-modified MBOs, the number of phosphorothioate linkages can be reduced by introducing alternative phosphodiester and phosphorothioate linkages in a 2′-*O*-methylribonucleoside segment without compromising the nuclease stability [101]. These end-modified MBOs have produced significantly less prolongation of aPTT [101], suggesting that by careful balance, the number of phosphorothioate linkages can be reduced to minimize protein binding without affecting in vivo disposition.

11. Centrally modified MBOs

In centrally modified MBOs, the modified oligonu-

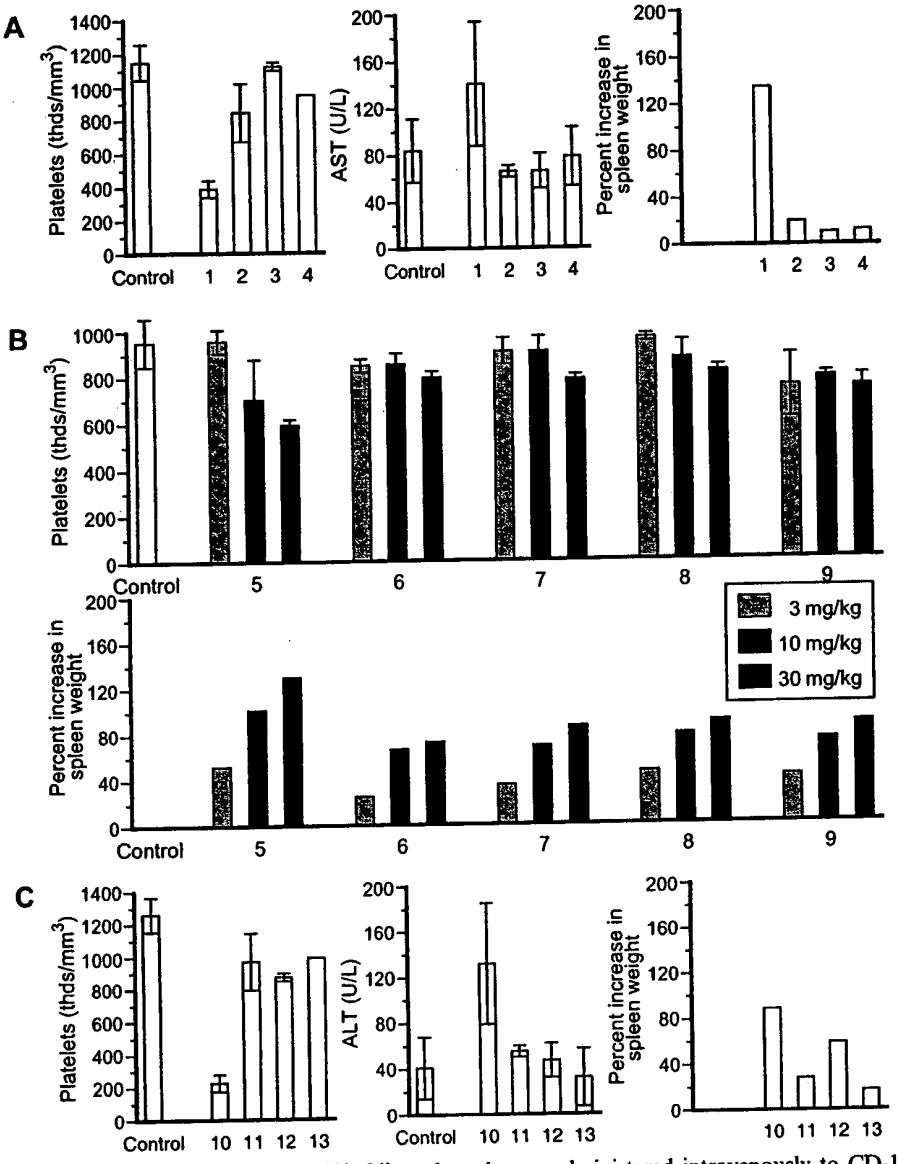

Fig. 3. Toxicity of oligonucleotides in mice and rats. (A) Oligos **1** to **4** were administered intravenously to CD-1 mice at a dose of 15 mg/kg daily for 7 days. On day 8, mice were killed and their spleens removed and weighed. Blood samples were taken for platelet counts and levels of serum aspartate aminotransferase (AST). Oligo **1** caused a decrease in platelet count and an increase in AST levels and spleen weight. Modification of the CpG motif in oligos **2**, **3**, and **4** resulted in minimization of these side effects. (B) Oligos **5** to **9** were administered intravenously to Fischer-344 rats at doses of 3, 10, and 30 mg/kg daily for 7 days. On day 8, the rats were killed and their spleens removed and weighed. Blood samples were taken for platelet counts. Oligo **5** caused a dose-dependent decrease in platelet count and an increase in spleen weight. Modification of the CpG motif is in oligos **6**, **7**, **8**, and **9** had some minimization of these side effects. (C) Oligos **10** to **13** were administered intravenously to CD-1 mice at a dose of 30 mg/kg daily for 7 days. Samples were processed by the same procedure as in the case of A. Oligo **10** caused a decrease in platelet count and an increase in ALT levels and spleen weights compared to oligos **11**, **12**, and **13**, suggesting that flanking sequence and site of CpG motif in PS-oligo is critical for its impact on toxicity. For details of the above protocol, please refer to Agrawal et al. [60].

cleotide is incorporated in the center of the PS-oligo [82,83]. The main advantage of centrally modified MBOs over PS-oligos is that they have few polyanionic-related side effects because they have shorter segments of PS-oligos [82,83]. In addition, oligonucleotides (including 2′-*O*-alkylribonucleotides) containing phosphodiester linkages can be incorporated. Because of the presence of PS-oligos at both the

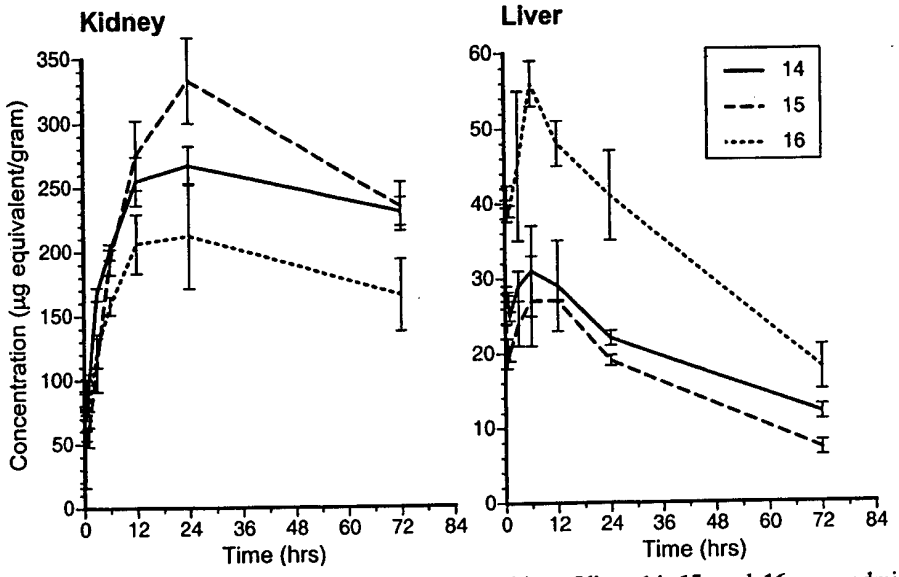

Fig. 4. Impact of number of phosphorothioate linkages on tissue disposition. Oligos **14**, **15**, and **16** were administered to mice at a dose of 30 mg/kg, intravenously. At various time points, animals were killed and various pharmacokinetic parameters were studied (see [83] for details). Disposition of oligo **14**, **15**, and **16** in kidney and liver showed that oligo **14** and **15**, which have reduced phosphorothioate linkages, had increased accumulation in kidney and decreased accumulation in liver compared to oligo **16** which is completely phosphorothioate. Also, increased urinary elimination was observed with oligos **14** and **15** compared to oligo **16** [83].

3′- and 5′-ends, the centrally modified oligonucleotide region is not exposed to exonucleases and therefore remains intact [83]. We have studied centrally modified MBOs containing methylphosphonate [82,83] and 2′-*O*-methylribonucleosides containing phosphorothioate and phosphodiester linkages, and have observed improvements over PS-oligos in many respects [82,83].

In general, MBOs have provided encouraging results over PS-oligos and are being explored for their therapeutic potential in human clinical trials [99]. Other modifications are being explored as well, to further improve the therapeutic potential.

12. Structural modification of PS-oligos

To improve the nuclease stability and minimize the polyanionic nature of oligonucleotides, self-stabilized oligonucleotides have been developed [102,103]. These self-stabilized oligonucleotides are PS-oligos that contain a hairpin structure at the 3′-end. This hairpin structure gets destabilized in the presence of target RNA, thereby allowing the antisense oligonucleotide to bind to target mRNA. Self-stabilized oligonucleotides have been studied for their biophysical, biochemical, and biological activities [102,103], and their pharmacokinetic [104] and safety profiles [61].

13. Bio-reversible analogs of PS-oligos

To improve cellular uptake and minimize undesirable polyanionic-related side effects, we have synthesized and studied PS-oligos that have been derivatized by attaching an acyloxyalkyl group to the internucleotide sulfur moiety; these are referred to as pro-drugs of oligos [105–107]. Incubation of pro-drugs of oligos with esterase bio-reverts the pro-drug to the parent PS-oligo. The rationale behind the use of a pro-drug is that by using the appropriate parent ester group, it would be possible to design pro-drugs for sustained release, site-specific targeting, and oral bioavailability, in addition to above mentioned advantages.

14. Delivery of oligonucleotides

It is important to note that while the sequence of oligonucleotides and its modification are important

factors in antisense activity, delivery of oligonucleotides, including tissue disposition, degradation, and elimination are also important factors for their efficacy in vivo. Oligonucleotides containing phosphodiester internucleotide linkages are rapidly degraded following their administration in vivo [52]. PS-Oligos have increased stability and are widely distributed to major tissues [51,52]. End-modified MBOs containing 2′-*O*-methylribonucleosides and phosphorothioate linkages have shown tissue disposition in animals similar to that observed with PS-oligos, and a significant increase in stability has been achieved [95]. Binding of PS-oligos to serum protein serves as a reservoir, saturation of which results in rapid elimination of PS-oligos in urinary excretion [51]. Aspirin, which affects PS-oligos by binding to serum proteins, has been shown to alter the pharmacokinetics [108]. Reduction in serum protein binding of oligos by introduction of non-ionic linkages (e.g., methylphosphonate) or with increased phosphodiester linkages (e.g., 2′-*O*-methylribonucleotides) has also resulted in increased disposition of administered oligonucleotide to kidney and elimination in urinary excretion [83]. By careful balance of the number of phosphorothioate linkages and other modified oligonucleotides, the rate of elimination and preferential tissue disposition can be achieved [101]. Oligos **14** and **15** (Table 1), which have fewer phosphorothioate linkages, were found less in the liver and more in the kidney than was oligo **16**, which contained all phosphorothioate linkages [83]. Similar results have been obtained with PS-oligos containing 2′-*O*-propylriboncleosides and 2′-*O*-methoxyethoxyribonucleosides containing phosphodiester backbone [58,100] (Fig. 4).

In addition to parenteral route for oligonucleotide administration, we also explored non-parenteral routes, including oral and colorectal [96,97]. PS-Oligos, when administered to mice by gavage, showed good metabolic stability in the stomach; however, extensive degradation was observed in the lower part of the gastrointestinal tract [96,97]. End-modified MBOs containing either 2′-*O*-methylribonucleosides with phosphorothioate linkages or MBOs containing methylphosphonate internucleotide linkages showed improved metabolic stability in the stomach and lower part of the gastrointestinal tract compared to PS-oligos. Tissue disposition studies in mice showed that end-modified MBOs were absorbed when administered by oral gavage and were distributed to major tissues. Similar results of absorption have been obtained following colorectal administration of end-modified MBOs [97].

The bioavailability of a drug is generally calculated based on the concentration of the drug in the plasma compartment versus time (area under the curve (AUC)) [109–111]. Oligonucleotides have short plasma residence times and are rapidly cleared to and retained by the tissues. The half-life of the oligonucleotide in plasma depends on serum protein binding and saturation, which may alter the distribution significantly. Based on our experience with various oligos, we have reached the conclusion that plasma pharmacokinetic parameters do not provide the whole picture in terms of bioavailability in the case of oligonucleotides. Concentration of oligonucleotides in tissues should also be considered when calculating the bioavailability.

15. Future directions

Rapid strides are being made in understanding the rules that govern the effective use of antisense oligonucleotides. It is clear that oligonucleotides can exert biological effects by multiple mechanisms, and the therapeutic potential of oligonucleotides can be explored based on these mechanisms. The sequence of an oligonucleotide is one of the key factors in controlling its mechanism of action and specificity. Selected oligonucleotide sequences can be appropriately modified to enhance the desirable properties and minimize the undesirable properties for their intended uses. Ongoing studies with second-generation oligonucleotide MBOs will further guide us in improving therapeutic potential of antisense oligonucleotides. These modified oligonucleotides which have specific mechanism of action can be used widely for gene target validation as well.

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